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A method for detecting variations in serum total bilirubin, said method comprising the step of detecting variation in a gene encoding UDP-glucuronosyltransferase (UGT).

Preferably the method comprises detecting variation in the gene encoding UGT1*1.

By gene is meant, the coding and noncoding sequences and upstream and downstream noncoding regions.

In one embodiment the invention comprises the steps of amplifying an upstream promoter region of the UGT1*1 exon 1 in blood and identifying the products of the amplification reaction.

Preferably the upstream promoter region is amplified using a polymerase chain reaction (PCR).

According to the invention there are provided suitable primers for the PCR reaction including primer pairs A/B (A,5'-AAGTGAACTCCCTGCTACCTT-G', B,5'-CCACTGGGATCAACAGTATCT-3') or C/D (C,5'-GTCACGTGACACAGTCAAAC-3'; D 5'-TTTGCTCCTGCCAGAGGTT-3')

The invention further provides a method for detecting individuals having Gilbert Syndrome or other diseases characterised by unconjugated hyberbilirubinaemia.

The invention further provides use of a method as defined herein in screening individuals prior to drug trials.

/u/mur/recordals/r17218

Summary

We have examined the variation in the serum total bilirubin (STB) concentration in a representative group of the Eastern Scottish population (drug-free, alcohol-free non-smokers) in relation to genotype at the UDP-glucuronosyltransferase subfamily 1 (UGT1) locus. Subjects with the 7/7 genotype in this population have a significantly higher STB than those with 6/7 or 6/6 genotypes. Of 14 control subjects who underwent a 24 hour fast to establish whether they had Gilbert Syndrome (GS), only 7/7 subjects had GS. In addition, one confirmed GS patient, two recurrent jaundice patients and 9 clinically diagnosed GS patients had the 7/7 genotype. Segregation of the 7/7 genotype with elevated STB concentration has also been demonstrated in a family with 4 Gilbert members. The incidence of the 7/7 genotype in the population is 10-13%. Here, we demonstrate a correlation between variation in the human STB concentration and genotype at a TATA sequence upstream of the UGT1*1 exon 1 and that the 7/7 genotype is diagnostic for GS.

Introduction

Gilbert Syndrome (GS) is a benign unconjugated hyperbilirubinaemia occurring in the absence of structural liver disease and overt haemolysis and characterized by episodes of mild intermittent jaundice. It is part of a spectrum of familial unconjugated hyperbilirubinaemias including the more severe Crigler-Najjar (CN) syndromes (types 1 and 2). GS is the most common inherited disorder of hepatic bilirubin metabolism occurring in 2-12% of the population and is often detected in adulthood through routine screening blood tests or the fasting associated with surgery/intercurrent illness which unmasks the hyperbilirubinaemia[1-3]. The most consistent feature in GS is a deficiency in bilirubin glucuronidation but altered metabolism of drugs has also been reported[3-5]. Altered rates of bilirubin production, hepatic haem production and altered hepatic uptake of bilirubin have been reported in some GS patients[2]. Due to the benign nature of the syndrome and its prevalence in the population it may be more appropriate to consider GS as a normal genetic variant[2] exhibiting a reduced bilirubin glucuronidation capacity (which in certain situations such as fasting, illness or administration of drugs) could precipitate jaundice.

The inheritance of GS has been described as autosomal dominant or autosomal dominant with incomplete penetrance based on biochemical analysis[6]. More recent reports have suggested

that the mildly affected (Gilbert) members of families in which CN type 2 (CN-2) occurs are heterozygous for mutations in the UDP-glucuronosyltransferase subfamily 1 (UGT1) gene which cause CN-2 in the homozygous state. The inheritance of GS in these families is autosomal dominant while CN-2 is autosomal recessive[7-11]. However, the incidence of CN-2 in the population is very rare and the frequency of alleles causing CN-2 would not be sufficient to explain the population incidence of GS.

An abstract by Bosma et al[12] suggested a correlation between homozygosity for a 2bp insertion in the TATA box upstream of UGT1*1 exon 1 and GS (no mutations were found in the coding sequence of the UGT1*1 gene). In this report we demonstrate that the primary genetic factor contributing to the variation in the serum total bilirubin (STB) concentration in the Eastern Scottish population is the sequence variation reported by Bosma et al[12]. In addition, we show that the 7/7 genotype is associated with GS and occurs in 10-13 % of the population.

Methods

Patients and Controls

Whole blood (3ml) was collected into EDTA(K3) Vacutainer tubes (Becton Dickinson) from one confirmed male Gilbert patient (diagnosed following a 48 hour restricted diet[13]), two female patients with reccurrent jaundice/associated elevated STB (29-42 μmol/l) and 9 (1 female, 8 male) clinically diagnosed GS subjects (persistent elevation of the STB amidst normal liver function tests). The patients were aged 22-45 years.

77 non-smoking residents selected at random from the Tayside/Fife region of Scotland (39 females aged 19-58 years, mean 32.41 $\pm$ 10.94; 38 males aged 23-57, mean 35.58 $\pm$ 9.04) participated in this study. Whole blood (9ml) was collected (8-10am) into EDTA(K3) Vacutainer tubes (Becton Dickinson) for DNA extraction and SST Vacutainer tubes (Becton Dickinson) for biochemical investigations. The subjects had not taken any medication or alcohol in the previous 5-7 days and had fasted overnight (12 hours). 14 controls subsequently underwent further biochemical tests (following a 3 day abstinence from alcohol) before and after a 24 hour 400-calorie diet[14] to determine if they had GS. All patients/controls were fully informed of the study and gave consent for their blood to be used in this study with the approval of the Tayside Committee on Medical Ethics.

Biochemistry and DNA Extraction

The following biochemical tests were performed on control blood samples: alanine aminotransferase, albumin, alkaline phosphatase, amylase, STB, cholesterol, creatinine, creatine kinase, free thyroxine, gamma-glutamyl-transferase, glucose, HDL-cholesterol, HDL-cholesterol/total cholesterol, iron, lactate dehydrogenase, percentage of saturated transferrin (PSAT), proteins, serum angiotensin converting enzyme, thyroid stimulating hormone, transferrin, triglycerides, urate, urea. 14 controls also had pre- and post-fasting (24 hour) alanine aminotransferase, albumin, alkaline phosphatase, STB and urate measured. DNA was prepared using the Nucleon II Genomic DNA Extraction Kit (Scotlab) according to manufacturer's instructions.

Genotyping

Polymerase Chain Reaction

Primer pairs A/B (A, 5′-AAGTGAACTCCCTGCTACCTT-3′; B, 5′-CCACTGGGATCAACAGTATCT-3′) or C/D (C, 5′-GTCACGTGACACAGTCAAAC-3′; D, 5′-TTTGCTCCTGCCAGAGGTT-3′) flanking the TATA box sequence upstream of the UGT1*1 exon 1 were used to amplify fragments of 253-255bp and 98-100bp, respectively. Amplifications (50μl) were performed in 0.2mM of each deoxynucleoside triphosphate (dATP, dCTP, dGTP, dTTP), 50mM KCl, 10mM Tris.HCl (pH 9.0 at 25°C), 0.1% Triton X-100, 1.5mM $MgCl_2$, 0.25μM of each primer, 1 Unit of Taq Polymerase (Promega) and human DNA (0.25-0.5μg). The polymerase chain reaction (PCR) conditions using the Perkin-Elmer Cetus DNA Thermal Cycler were: 95°C 5min followed by 30 cycles of 95°C 30 sec, 58°C 40 sec, 72°C 40 sec.

Direct Sequencing

Amplification was confirmed prior to direct sequencing by agarose gel electrophoresis. Sequencing was performed using [α-^{35}S]-dATP (NEN Dupont) with the USB Sequenase™ PCR Product Sequencing Kit according to manufacturer's instructions. Sequenced products were resolved on 6% denaturing polyacrylamide gels. The dried gels were exposed overnight to autoradiographic film prior to developing.

Radioactive PCR

Amplification was performed as above using primer pair C/D except that 2.5 pmol of primer C was radioactively 5' end-labelled with 2.5μCi of [γ-^{32}P]-ATP (NEN Dupont) prior to amplification. Products were resolved on 6% denaturing polyacrylamide gels and the wet gels exposed to autoradiographic film (-70°C 15 min) and the autoradiographs developed.

Statistics

A t-test was used to determine if there was a significant age difference between males and females. χ^2 analysis was used to assess any difference in the distribution of the 6/6, 6/7 and 7/7 genotypes in males and females and also to determine if the 7/7 subjects from the 24 hour fasted group had STB elevated into the range diagnostic for GS[14]. An analysis of variance was performed to compare mean STB in males and females within each genotype group. A non-parametric test, the Mann-Whitney U-Wilcoxon Rank Sum W Test was used to determine whether there was a significant difference in mean STB between males and females (irrespective of genotype). Correlations and significance tests were performed for STB versus PSAT and STB versus iron. A probability (p) of < 0.05 was accepted as significant.

Results

There was no significant age difference between males and females ($t = -1.38$, $p = 0.17$). Genotypes were determined initially by amplification/sequencing and later by the radioactive PCR approach. Individuals homozygous for the common allele, heterozygous or homozygous for the rarer allele have the genotypes 6/6, 6/7 and 7/7, respectively. 12 DNA samples (2 of 6/6, 3 of 6/7 and 4 of 7/7) were analysed by both methods and genotype results were identical (see Figure 1).

Genotype frequencies in male controls were 6/6 (44.74%), 6/7 (44.74%), 7/7 (10.52%) and in female controls were 6/6 (35.9%), 6/7 (51.3%), 7/7 (12.8%). There was no significant difference between the genotype proportions in the two groups ($\chi^2 = 0.6$ at 2 df, $p = 0.7$). Control h (encircled in Figure 2) had a STB which was 2.4 SD above the mean STB for that group (mean calculated including control h). The results for control h were repeatable and he is currently being investigated to exclude haemochromatosis. Comparison of mean STB in males

and females revealed that females have a significantly lower concentration than males ($p = 0.031$ including control h; $p = 0.0458$ excluding control h). There was a strong correlation between genotype and mean STB concentration within the control group ($p < 0.001$) irrespective of whether control h was included and there was a significant difference in mean STB between males and females of the same genotype ($p < 0.05$) irrespective of whether control h was included (see Figure 2). All patients studied had the 7/7 genotype.

Correlations between STB/PSAT ($r = 0.4113$, $p = 0.001$)(see Figure 2) and STB/iron ($r = 0.4712$, $p = 0.001$) were observed. The correlation between STB and iron was stronger in females ($p = 0.001$) than males ($p = 0.01$) but when control h is excluded there was no significant correlation in males.

The STB concentrations of controls who underwent the 24 hour restricted diet (see Methods) are shown in Table 1. The normal fasting response is a small rise in the base-line STB (not exceeding a final concentration of 25μmol/l) most of which is unconjugated while GS patients have as a lone biochemical feature a raised STB (>25μmol/l but <50μmol/l) most of which is unconjugated[14]. The 6/6 and 6/7 controls had post-fasting STB of ≤ 23μmol/l while all 7/7 controls were ≥ 31μmol/l. Other liver function tests were within acceptable ranges for the age and sex of the subjects. The 7/7 genotype correlates with a fasted STB (24 hour) within the range diagnostic for GS[14] ($p < 0.01$)(see Table 1). In addition, the 7/7 genotype segregates with elevated STB concentration in a family with 4 GS members (Figure 3).

Discussion

A few recent reports claim to have identified the genetic cause of GS[10-12]. Clinical diagnosis of GS is often based on a consistent mildly elevated non-fasting STB (>17 μmol/l) as the sole abnormal liver function test, intermittent jaundice or both. The diagnosis can be confirmed by elevation of the STB to 25-50μmol/l after a 24 hour 400-calorie diet[14] or by elevation of the unconjugated bilirubin by > 90% within 48 hours of commencing a 400 calorie diet[13].

Sato's research group recently reported the occurrence of 7 different heterozygous missence mutations in unrelated Gilbert patients (most of the mutations have been found in the homozygous state in affected members of CN families), however, the non-fasted STB for these patients were > 52μmol/l (with the exception of one, 31μmol/l)[10,11]. These non-fasted STB

concentrations already exceed the diagnostic range for GS[14], hence these patients have a more severe form of hyperbilirubinaemia than those studied in this report, while those in the Bosma et al[12] abstract had STB concentrations similar to those studied here.

In this study we show that the variation in the STB levels after an overnight fast (and in the absence of exposure to known inducers of the UGT1*1 isoform in GS, such as alcohol[15] and drugs[16]) in a representative group of the Eastern Scottish population is primarily due to (or associated with) the TATA box sequence variation reported by Bosma et al[12]. In agreement with previous work females have a significantly lower mean STB concentration than males[17,18].

Individuals with the 7/7 genotype in the population have GS (see Table 1). One of the 7/7 controls indicated in Table 1 had a non-fasting STB similar to those reported for heterozygous carriers of CN-2 mutations[7-11] which suggests that this subject may also be a carrier of a CN-2 mutation, alternatively, the very elevated bilirubin in this patient may be due to the coexistence of Reavon's Syndrome (characterized by a collection of abnormal biochemical results which are risk factors for coronary heart disease)[19].

We have found that 10-13% of the Eastern Scottish population have the genotype associated with mild GS. None of the Gilbert subjects from the control population were aware that they had an underlying metabolic defect in glucuronidation which testifies to its benign nature. Three 7/7 controls had STB concentrations comparable to mean levels observed in heterozygotes, however, they also had a lower than normal PSAT ($\leq$ 22%)(see Figure 2). The observed correlation between STB and PSAT ($p = 0.001$)(Figure 2) and STB and iron (females $p = 0.001$ and males $p = 0.01$ including control h) indicates that other genetic and environmental factors affecting the serum PSAT and iron values will in turn affect the STB concentration.

From the data presented here and previous reports it seems clear that there are mild and more severe forms of GS. The milder form (fasted STB 25-50μmol/l) is either caused by (or is associated with) a homozygous 2bp insertion at the TATA sequence upstream of the UGT1*1 exon 1 (autosomal recessive inheritance) while the rarer more severe dominantly inherited forms identified to date[7-11] (non-fasted STB $>$ 50μmol/l) are due to heterozygosity for a mutation in the coding region of the UGT1*1 gene which in its homozygous state causes CN-2. The particular genetic abnormality causing GS in a patient will have implications for genetic counselling as the dominantly inherited form in two GS patients could result in offspring with

CN-2, whereas the recessive form in one or both GS patients would have less serious implications. It is important to disciminate between the two forms and provide suitable genetic counselling for such couples. The rapid DNA test presented here (less than 1 day for extracted DNA) carried out in addition to biochemical tests following a 12 hour overnight fast (without prior alcohol or drug intake, see Methods) would permit such a diagnosis. The compliance rate for the current 24 and 48 hour restricted diet tests for GS[13,14] is debatable and hence the overnight fast has obvious advantages and only one blood sample is required (for genetic and biochemical analysis) in contrast to the 2-3 blood samplings required for the 24 and 48 hour tests. This approach to GS testing would be cost effective in terms of fewer patient return visits to clinics and in identifying couples at risk of having children with CN-2.

In addition, the recent finding of an increased bioactivation of acetominophen (a commonly used analgesic which is eliminated primarily by glucuronidation) in GS patients indicates the greater potential for drug toxicity in these patients if administered drugs which are also conjugated by UGT1 isoforms[3]. In fact, ethinylestradiol (EE2) has recently been shown to be primarily glucuronidated by the UGT1*1 isoform in man[20] and hence this could have implications for female Gilbert patients taking the oral contraceptive who are then more predisposed to developing jaundice.

Acknowledgements

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Figure 1

Genotypes at the TATA box sequence upstream of the UGT1*1 exon 1 determined by direct sequencing and radioactive PCR. A photographic representation of the sense DNA sequences obtained by PCR/direct sequencing of DNA samples having the genotypes 6/6, 6/7 and 7/7 from left to right, respectively. The common allele, $(TA)_6TAA$, is denoted by "6" while the rarer allele, $(TA)_7TAA$, is denoted by "7". Below each sequence is a photographic representation of the 98 100bp resolved fragments amplified using primer pair C/D which flank the TATA sequence upstream of the UGT1*1 exon 1. The additional fragments of 99 and 101 bases (indicated by the arrows) are thought to be artfacts of the PCR process where there is non-specific addition of an extra nucleotide to the 3' end of the amplified product[21].

Figure 2

Serum total bilirubin (μmol/l) plotted against UGT1*1 exon 1 genotype. Males (M) and females (F) are plotted separately. Each circle/square represents the result of a single control

subject. The squares indicate the 14 controls who also underwent the 24 hour restricted diet (see Methods). The filled circles/squares represent those who had a lower than normal PSAT (≤ 22%) while the half-tone circles represent those who had a higher than normal PSAT (≥ 55%). The mean STB concentrations (indicated by the horizontal lines) for males were 13.24 ± 3.88 (6/6), 13.94 ± 6.1 (6/7) including control h or 12.69 ± 3.34 excluding control h, 29 ± 14.45 (7/7) and for females were 9 ± 3.62 (6/6), 12.2 ± 3.53 (6/7), 21.6 ± 7.8 (7/7). The encircled result is from control h (discussed in the text).

Figure 3

Segregation of the 7/7 genotype with elevated serum total bilirubin concentration in a family with GS. Males and females are represented by squares and circles, respectively. Filled and half-filled circles/squares indicate the genotypes 7/7 and 6/7, respectively. The numbers in parentheses below each member of the pedigree are the STB concentrations measured after a 15 hour fast and 7 day abstinence from alcohol. All family members were non smokers who were not taking any medication when the biochemical tests were performed. Elevated STB are underlined. Individual members of each generation (I or II) are denoted by the numbers 1-4 above each circle/square.

Table 1

Comparison of the UGT1*1 exon 1 genotype with elevation in the serum total bilirubin after a 24 hour 400-calorie restricted diet[14]. An elevation of the fasting STB to a final concentration in the range 25-50μmol/l is considered to be diagnostic for GS[14]. The 7/7 subject denoted by * has a fasting and non-fasting STB of > 50μmol/l but this value is within a range considered by others to conform to a diagnosis of GS[7-11].

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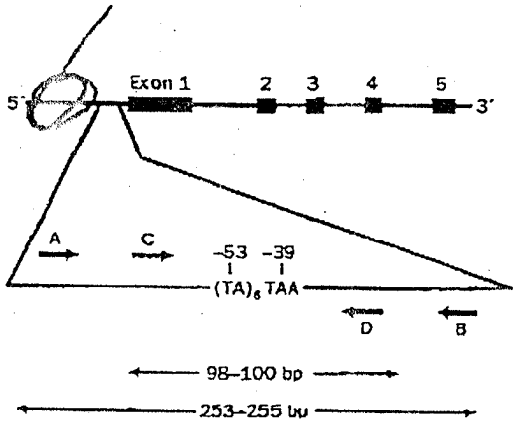

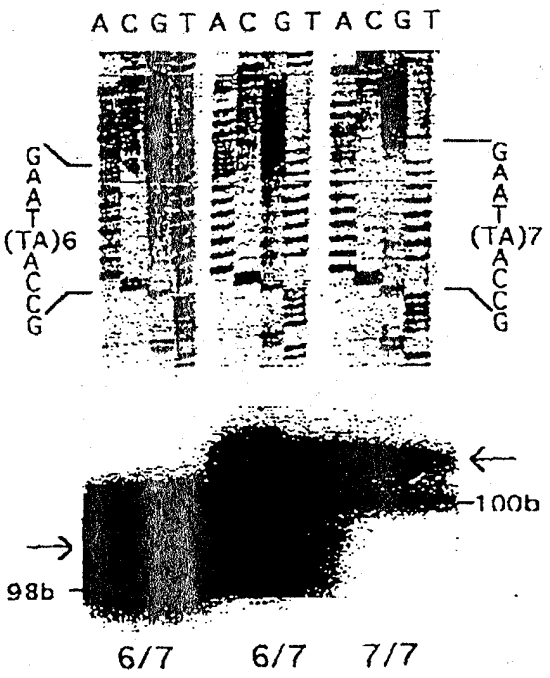
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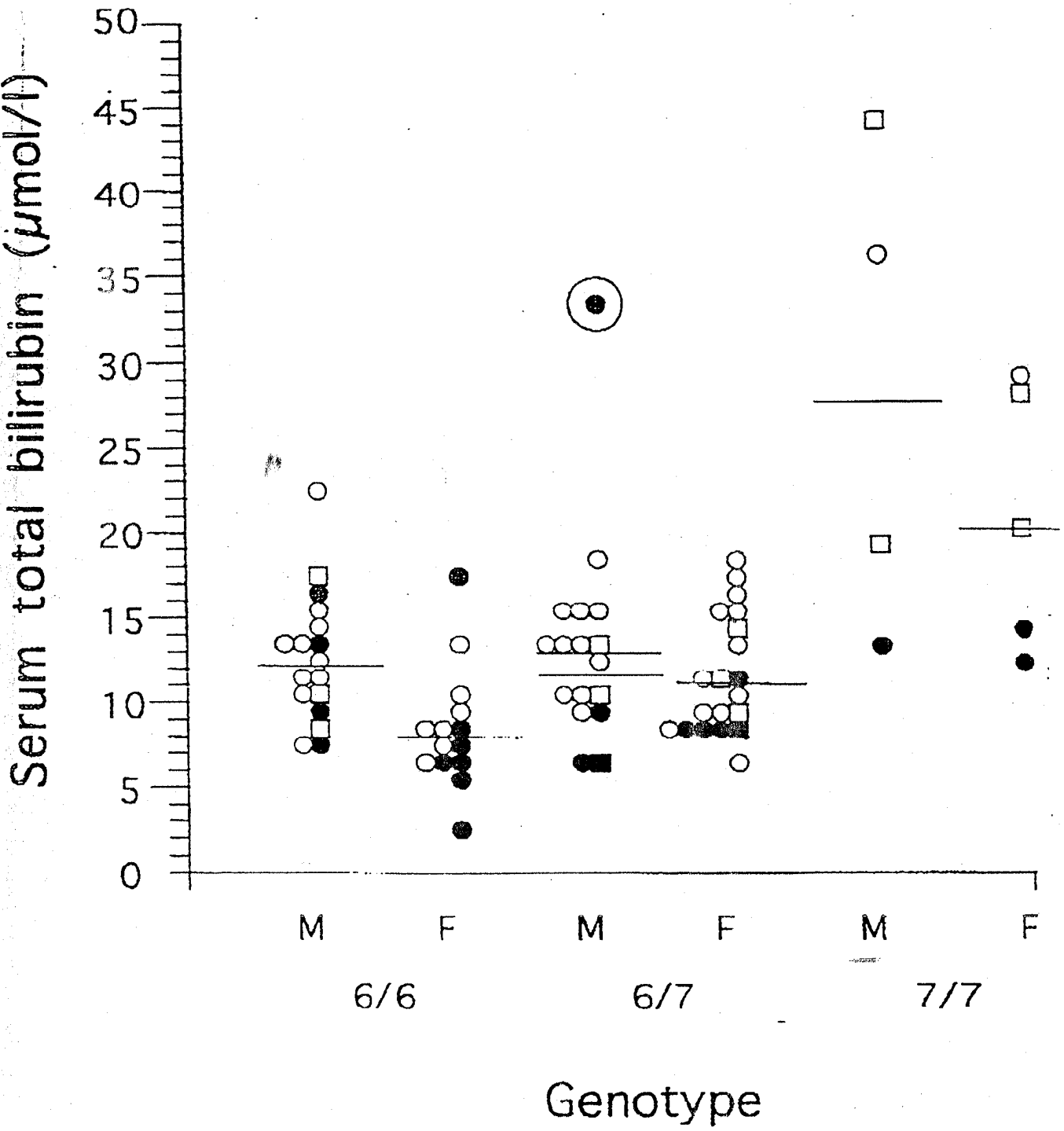
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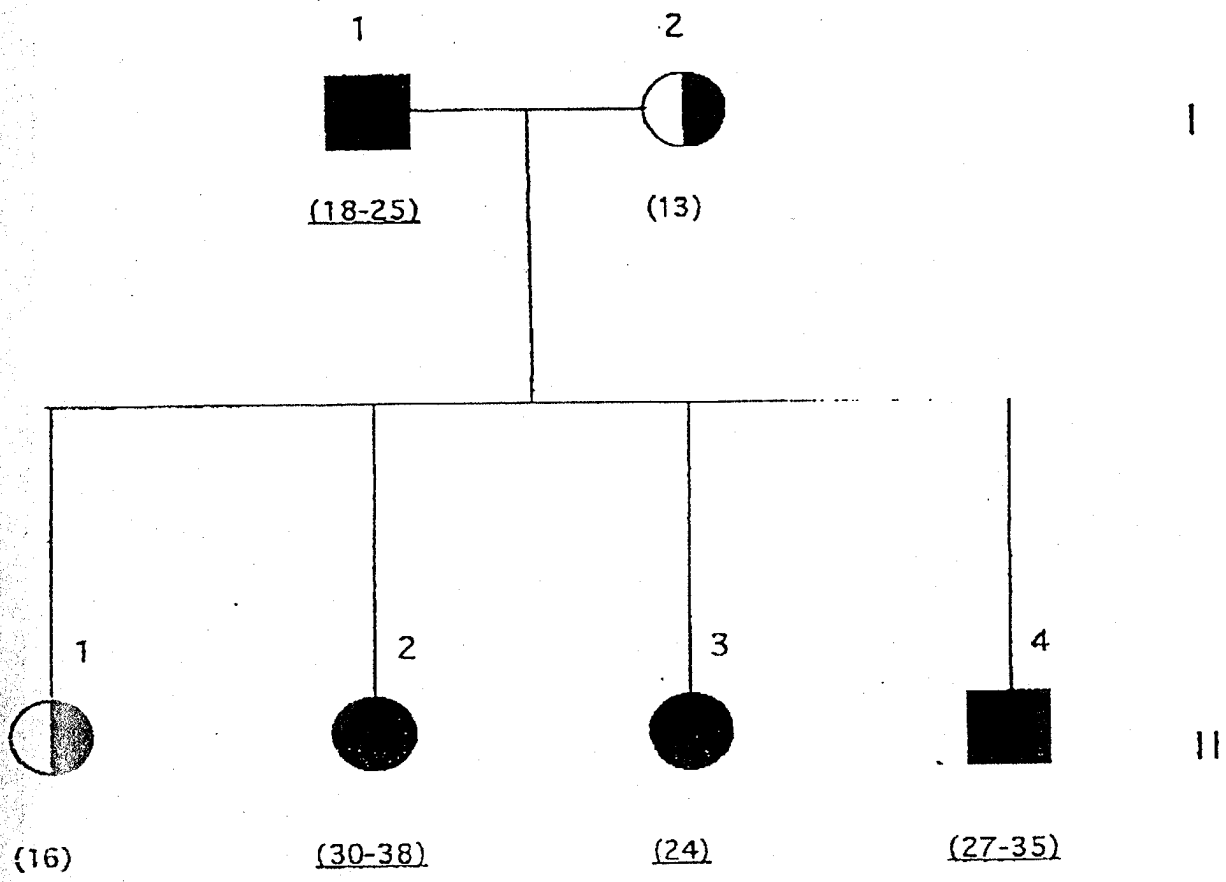
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II
(16)
(30-38)
(24)
(27-35)

Genotype	Sex	24 hour fast		Fasting bilirubin >25 & <50μmol/l
		Before	After	
6/6	M	8	17	NO
	M	9	19	NO
	M	12	15	NO
6/7	F	8	17	NO
	F	9	13	NO
	F	11	12	NO
	F	12	17	NO
	M	8	10	NO
	M	15	23	NO
	M	17	18	NO
7/7	F	9	34	YES
	F	12	34	YES
	M	19	31	YES
	M	62	96	NO*